Internalization of uranium-citrate complexes in Helianthus annuus and a role of iron transporter in uranium uptake and translocation in Arabidopsis.

STATE OF ART

Low molecular organic acids (LMOA) play an important role in the mobilization of nutrients in soil and their transport into/in plants (1, 2, 3). Currently, some heavy metals are released into soil solution and their bioavailability for plant increases.
Uranium as uranyl is poorly available for plants and an overwhelming majority of it is precipitated in roots: in the extracellular space more often in phosphate (4, 5). LMOA enhances the U translocation about 10 – 1000 orders (6). Undoubtedly, citric acid (CA) is one of the most effective (7, 8). The model of uranium speciation in the presence of CA predicts a domination of U-citrate complexes (9). Several hints tend to the hypothesis that U does not change its speciation as passes into root cells and rests in citrate complexes (10). Subsequently, this species is translocated from a stele by xylem into leaves.
Robbard and Rodds (11) devoted their attention to the U internalization in plant cells by endocytosis.
Straczek et al (12) recorded a difference between monocot and dicot plant in a radial distribution of U in root. More homogenous U distribution in roots of dicot plants and its present in a stele point on the U translocation through symplast.
Gunther et al. (5) and Laurette et al. (13) reported about the presence of U-phosphate in the form of meta-autunite in plant tissues.
Laurette et al. (10) speculated about diffuse of neutral and negatively charged complexes through the symplast, without being captured in root cell vacuoles. Moreover, the U vectorization to plant shoot after CA administration they directly assigned to citrate.
In this point, it is important to note several links between iron and uranium mentioned below.

THE OBJECTIVES OF THE RESEARCH

Several hints allow us to hypotized that the mechanism of uranium uptake and translocation after the citric acid treatment is similar to the iron's mechanism (12, 14, 15).
Generaly, there are following links between iron and uranium:
- both are availabile for plants as a bivalent ions,
- they form strong complexes with citric acid that further carries (at least) iron into leaves,
- iron membrane transporters are not completly specific and work for other bivalent metals as well.
The efficiency of citric acid on uranium translocation into leaves is well known but not so processes that uranium must undergo.
We want to reveal a) the role of the iron membrane transporter (Iron-Regulated Transporter1 - IRT1 and Ferric Reductase Deficient3 - FRD3) in uranium translocation from roots to shoots, b) the role of citric acid in that translocation.
Can uranium employ the iron membrane transporters?
Does the exogenous citric acid act as a carrier for uranium in the translocation to xylem or only in root apoplast?
In order to elucidate those phenomenons our proposal contains parallel branches:
a) Verifying the role of iron transporters by the growing of Arabidopsis thaliana mutants deficient in iron membrane transporters,
b) Administration of 14C-citric acid for confirmation of the uptake and translocation of uranium-citrate complexes.
To confirm the uranium-citrate complexes through symplastic pathway the use of 14C labeled citric acid is appropriate. The presence of the labelled CA in xylem will be proof of the uptake and the direct translocation of CA or U-citrate complex into the xylem. The beta emision after elution of unlike compounds to citric acid will signal the transformation of citric acid in cells.
The sunflower (Helianthus annuus) will be employed in these experiments due to its accumulation of heavy metals in roots and our previous experiences with the U translocation (15).

The choice of Arabidopsis thaliana is based on a) the existence and commercial availability of the IRT1 and FRD3 mutants (Arabidopsis Biological Resource Center), b) their ability to accumulate a well measurable quantity of U in leaves (14, 16).
Moreover, never before has the uranium speciation in transient state been analysed after the citric acid treatment. The challenge is also to combine more analytical technics for determining the labeled citric acid.

Hypothesis
FRD3 as a transporter of iron is more likely able to transport citrate through cell membrans (17, 18). It is hypothesized often for iron uptake. The proof of the citrate as an iron carrier from exogenous space into the xylem will be provided by the labeled citric acid. An increase of the labeled citric acid in xylem will confirm its entrance from exogenous solution.
If citric acid is a carrier of iron and uranium from exogenous solution to xylem, we can suppose the current appearance of the elevated iron and uranium concentration in the fraction of the C14-citric acid determined by scintilator.
Finally, there is an option that exogenous uranium (iron)-citrate complexes are dissociated on the apoplast-symplast interface due to pH changes and later recreated entering into xylem (19). On the other hand, far bigger molecules like EDDS were traced from exogenous solution up to xylem (20).

A suppresed uranium uptake into the iron transporter deficient mutants will point to a role of these transporters in the uranium uptake. An increase of uranium in xylem in such mutants signals that uranium or uranium-citrate pass into xylem without dependecy on the function of these transporters. Vert et al. (21) showed that IRT1 is responsible for iron transport across membranes in root cells but not that for its translocation. Moreover, upon iron starvation, other bivalent metals like Zn, Mn, Co increase their concentration in the shoots. Knocking out of IRT1 transporters reduced the uptake of these metals similarly to iron (22).
Veihweger and Geipel (14) observed a drastic increase in uranium upon iron starvation in Arabidopsis plants, therefore, in our experimments there are also variants of the Arabidopsis growing under iron starvation.

Recent years have brought new outstanding results in the investigation of uranium uptake into plants (4, 10, 12, 13, 14) showing that ecotoxycological laboratories are still interested in bioavailability of uranium and they put more of their attention on the physiological and cell mechanism of uranium biovailability. However, the carrier and the pathway of uranium within plants are still hidden. The submitted proposal suggests the methods that aim to examinate the intra-plant uranium transport.

THE METHODOLOGY OF THE RESEARCH

Localization of 14C-citric acid in plant
Plant culture
Seeds of sunflower (H. annuus cv.) will be sterilized, saw on moistened sand and grown in a culture chamber at 25 °C and 65 % humidity under a 16/8 h photoperiod (150 microE m-2 s-1). One week later, seedlings will be transferred to polypropylene vials filled with sterile modified Hoagland's solution composed of 2 mM Ca(NO3)2, 2 mM KCl, 1 mM CaCl2, 1 mM K2SO4, 0.5 mM MgSO4, 0.4 mM KH2PO4, 0.2 mM FeNa-EDTA, 0.15 mM K2HPO4, 0.1 mM H3BO3, 5 microM MnCl2, 4 microM ZnSO4, 1 microM CuSO4, 1 microM Na2MoO4, 0.1 microM Co(NO3)2, pH 5.1. Three weeks old seedling will be relocated into a similar growth medium depleted of phosphat that will be administrated on leaves (8). Before the experiment we will need to carry out a preliminary trial in order to optimalized the age of plants sufficient for xylem collecting and time between the administration of CA and its increase in xylem.

Treatment:
a) the control group will grow in the basic solution and P will be administrated on leaves.
b) the group will grow in the basic solution and P will be administrated on leaves. 100 microM of UO2(NO3)2 will be added to the growth medium.
c) the group will grow in the basic solution and P will be administrated on leaves. 100 microM of UO2(NO3)2 and 10 microM of 14C-CA will be added to the growth medium.
d) the group will grow in the basic solution and P will be administrated on leaves. 100 microM of UO2(NO3)2 and 100 microM of 14C-CA will be added to the growth medium.

Each group contains five replicates.

Citric acid will be administrated one hour before switching on the light in phytotrone. Collecting of xylem sap will carry out approximately 3 hours later.

Harvesting and sampling
Plant shoots will be cut just below the first true leaf using a razor blade, and xylem sap will be left to exude. The sap of the first 5 min will be discarded to avoid contamination, the surface will be washed with distilled water and blotted dry, and sap will be then directly collected for 30 min using a micro-pipet and maintained in Eppendorf tubes kept on ice. Immediately after sample collection. Before analysis, samples will be thawed and diluted 2-fold with 10 mM ammonium acetate in methanol at pH 6.8. Then, samples will be vortexed, centrifuged at 12 000 g for 2 min and the supernatant immediately analyzed (23).

Analyses:
Organic acids present in xylem will be sequestered by the high pressure liquid chromatography (HPLC). Elution of 14C-citric acid from HPLC will be characterized by chromatograph calibrated with non-labeled citric acid. Further, the effluent of the HPLC will be conducted to a lithium-glass scintillator, which floresces in response to beta-rays. This method is illustrated in Mori (24) and could be innovated by a modern instrument like the Radiomatic 15TR flow scintillation analyzer (PerkinElmer).
The sequestered fractions, especially with the high beta-emission, will be analysed on iron and uranium by inductively coupled plasma mass spectrometry (ICP-MS).
If 14C-citric acid is metabolized in plant cells, we can expect the appearance of beta emision linked with elution some another compound.

Statistical evalution
Since the uranium-citrate complexes are uneasily determined a helpful tool for their revealing could be the correlation between the U concentration and the concentration of the labelled CA in xylem. Because, citric acid is carrier for iron as well, we must take it into consider.
Groups undergoing different treatment will be comparised with the help of ANOVA.
Results will be evaluated by statistical tools in R statistical software.

Uranium speciation in solution
Uranium speciation will be modeled using the software J-Chess (25).

Growing of Arabidopsis thaliana mutants
Plant culture
Seeds of A. thaliana (wild type Wasslewskija) and mutants irt1-1, frd3 will be sterilized, saw on moistened sand and grown in a culture chamber at 25 °C and 65 % humidity under a 16/8 h photoperiod (150 microE m-2 s-1). One week later, seedlings will be transferred to polypropylene vials filled with sterile modified Hoagland's (see above). Three weeks old seedling will be relocated into a similar growth medium depleted of phosphat.

Treatment
Two weeks old seedlings will be exposed to uranium and citric acid. The experiment is based on comparison the wild phenotyp of Arabidopsis (W) with ird1-1 mutant (IRD) and frd3 mutant (FRD). The basic groups of plants (W-0, IRD-0, FRD-0) will growt without exposure to uranium and exogenous citric acid.
The groups: W-1, IRD-1, FRD-1 will be exposed to uranium (concentration)
The groups: W-2, IRD-2, FRD-2 will be exposed to uranium and exogenous citric acid.
The groups: W-3, IRD-3, FRD-3 will suffer of iron starvation and be exposed to uranium.
The groups: W-4, IRD-4, FRD-4 will suffer of iron starvation and be exposed to uranium and citric acid.
Each group will contain 5 plants.

Analyses
Roots will be shortly rinsed with 10 % citric acid to remove the adherent U from the root surface(14). Plants will be separated into roots and shoots, dried and digested (0,5 g plant material) with HNO_3, H_2O_2, H_2O in a microwave.

Statistical evaluation will be computed by R statistic software.

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